ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1974, p. 420-425

Vol. 5, No. 4
Printed in U.S.A.

Studies on the Mechanism of Action of Miconazole: Effect of Miconazole on Respiration and Cell Permeability of *Candida albicans*

K. H. SREEDHARA SWAMY, M. SIRSI, AND G. RAMANANDA RAO

Microbiology and Pharmacology Laboratory, Indian Institute of Science, Bangalore-560012, India

Received for publication 20 December 1973

The antifungal drug, miconazole nitrate, inhibits the growth of several species of *Candida*. *Candida albicans*, one of the pathogenic species, was totally inhibited at a concentration of approximately 10 μg/ml. Endogenous respiration was unaffected by the drug at a concentration as high as 100 μg/ml, whereas exogenous respiration was markedly sensitive and inhibited to an extent of 85%. The permeability of the cell membrane was changed as evidenced by the leakage of 260-nm absorbing materials, amino acids, proteins, and inorganic cations. The results we present clearly show that the drug alters the cellular permeability, and thus the exogenous respiration becomes sensitive to the drug.

Miconazole nitrate [1-(2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl)imidazole nitrate] is a potent antifungal drug (4), the structure of which is shown in Fig. 1. The drug was obtained as a gift sample through the courtesy of Ethnor Ltd., Bombay, India. It is a white microcrystalline powder, has a molecular weight of 479, and is soluble in 50% ethanol on warming.

Miconazole has a broad spectrum of in vitro antimicrobial activity. It inhibits the growth of dermatophytes, namely, species of *Trichophyton*, *Microsporum*, and *Epidermophyton*, pathogenic and nonpathogenic yeasts, and gram-positive bacteria (4, 12). Its effective therapeutic use as a topical applicant in treating skin and nail infections in man and in vaginal candidiasis has been reported (2, 5, 16, 18). However, the mode of action of this drug has not yet been elucidated. In this paper we report our findings on the effect of miconazole on respiration and cell permeability of a pathogenic strain of *Candida albicans*, Z248.

MATERIALS AND METHODS

The microorganisms used in this study were obtained from the following sources: *Candida albicans* Z248, *C. parapsilosis* Z40, *C. pseudotropicalis* Z27, *C. krusei* Z70, *C. tropicalis* Z156, *C. pelliculosa* Z220, and *C. guilliermondii* Z55, Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine, London; *C. albicans* 502/9, *C. intermedia* 512/9 and *C. tropicalis* 502/7, V.P. Chest Institute, New Delhi, India. Cultures were maintained on Sabouraud glucose agar slants.

Cell growth. For growth studies both Sabouraud glucose medium containing 2% glucose and 1% Difco neopeptone, and synthetic medium containing 2% glucose, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% $CaCl_2 \cdot 2H_2O$, and 30 μg of biotin per liter were used. The inoculum was prepared by growing the cells in 250-ml Erlenmeyer flasks containing 100 ml of medium for 18 h on a rotary shaker (200 rpm) at 30 C.

Growth inhibition studies were carried out in 100 ml of Sabouraud or synthetic medium in 250-ml Erlenmeyer flasks with side arms. Required volumes of miconazole (dissolved in 50% ethanol) were added aseptically to the medium to give various drug concentrations. The flasks were inoculated with 0.1 ml of an 18-h culture of the organism grown in the same medium. Control flasks contained equal volumes of 50% ethanol. They were incubated at 30 C on a rotary shaker, and the growth was measured in a Klett-Summerson colorimeter using a no. 42 filter.

Cell viability. The viability of the cells was determined by serially diluting the samples with NaCl-peptone-water (0.5% NaCl, 0.1% peptone). A portion (0.1 ml) of the diluted sample was thoroughly mixed with 2 ml of molten soft agar (1.0% peptone, 0.5% NaCl, and 0.7% agar) at 45 C and poured over the surface of Sabouraud glucose agar plates. The plates were incubated for 48 h at 30 C.

Respiration studies. Studies on respiration were carried out using standard manometric techniques (17). Cells grown in Sabouraud glucose medium for 18 h were harvested and washed three times with physiological saline and suspended in 0.05 M phosphate buffer, pH 7.0. Warburg flasks contained in a final volume of 3.2 ml: 50 μmol of phosphate buffer, pH 7.0, 50 μmol of glucose, and 1 ml of cell suspension (12 mg dry weight) in the main compartment. The center well contained 0.2 ml of 20% KOH. In the side arm of

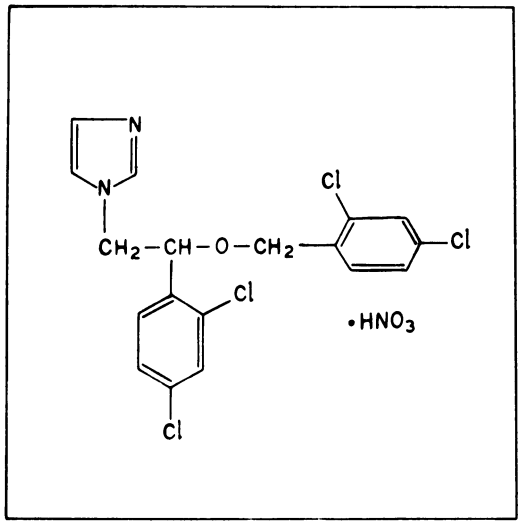

FIG. 1. *Structure of miconazole. 1-(2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl)imidazole nitrate.*

the flask, 0.5 ml of drug at appropriate concentration was taken and the control flasks contained 0.5 ml of 50% ethanol. The flasks were equilibrated for 10 min at 30 C, and the oxygen uptake was measured after the miconazole was tipped from the side arm.

Cell permeability studies. For these studies, cells were grown in Sabouraud glucose medium either in the presence or absence of [^{32}P]ortho-phosphoric acid (Bhabha Atomic Research Centre, Bombay, India) at a concentration of 0.4 μCi/ml. After 18 h of growth on a rotary shaker at 30 C, the cells were harvested and washed three times with distilled water and suspended in the same. The cells were exposed to miconazole at various concentrations on a rotary shaker at 30 C. At different time intervals samples were removed and the cell exudates were obtained by centrifugation. The cell exudates were examined for the 260-nm absorbing materials by measuring the absorbance at 260 nm, proteins were determined by the method of Lowry et al. (9), and amino acids were examined by the modified colorimetric method of Rosen (13). Potassium and sodium were estimated by flame photometry. The radioactivity in the cell exudates obtained from ^{32}P-labeled cells were determined in a Beckman LS-100 liquid scintillation counter.

RESULTS

Susceptibility of Candida species to miconazole. Various species of *Candida* have been screened for their susceptibility to the drug, and the minimum inhibitory concentration (MIC) values are shown in Table 1. Candida species markedly differed in their susceptibility to miconazole. Whereas *C. parapsilosis* and *C. pseudotropicalis* are highly susceptible, species like *C. pelliculosa, C. guilliermondii, C. intermedia,* and *C. tropicalis* needed 1,000-fold higher concentrations for their complete inhibition. In all the studies described below, a pathogenic strain of *C. albicans*, Z248, has been used. The growth patterns in Sabouraud and synthetic media and their inhibition by miconazole are shown in Fig. 2. At 10 μg/ml the growth was almost completely inhibited in Sabouraud medium, whereas at 1.0 and 0.1 μg/ml the effect was only partial. In the synthetic medium, the inhibitory effect of the drug was markedly reduced. Only 50% inhibition was noticed at 10 μg/ml.

Effect of miconazole on cell viability. The inhibition of growth of *C. albicans* in Sabouraud glucose medium was paralleled by a decline in the number of viable organisms (Table 2). The viability of cells is affected both by increasing the drug concentration and by prolonging the time of contact. At the 2.5 μg/ml level, more than 60% of cells were killed within 4 h, and at the end of 12 h of exposure the viability loss was 81%. Doubling the drug concentration had not increased the effect significantly. However, at 10 μg/ml, nearly 100% loss in viability was observed by 4 h.

Effect of miconazole on respiration. The effect of miconazole on endogenous respiration of unstarved and starved cells and exogenous respiration is shown in Fig. 3. The freshly harvested cells of *C. albicans* showed a very high level of endogenous respiration, and this level was not affected markedly by miconazole. Only at 100 μg/ml did it show a slight inhibition, and at 500 μg/ml it showed 30% inhibition. On prolonged shaking of the cell suspension for 6 h at 30 C on a rotary shaker, the endogenous respiration was markedly reduced. Even on these starved cells the drug failed to show any effect. When the endogenous respiration

TABLE 1. *Susceptibility of Candida species to miconazole*

Organism	MIC (μg/ml)[a]
Candida parapsilosis, Z40	0.01
C. pseudotropicalis, Z27	0.01
C. krusei, Z70	0.1
C. tropicalis, Z156	0.1
C. albicans, Z248	1.0
C. albicans, 502/9	1.0
C. pelliculosa, Z220	10.0
C. guilliermondii, Z55	10.0
C. intermedia, 512/9	10.0
C. tropicalis, 502/7	10.0

[a] The MIC was determined in Sabouraud glucose broth by serial tube dilution method. All the values are from duplicate determinations.

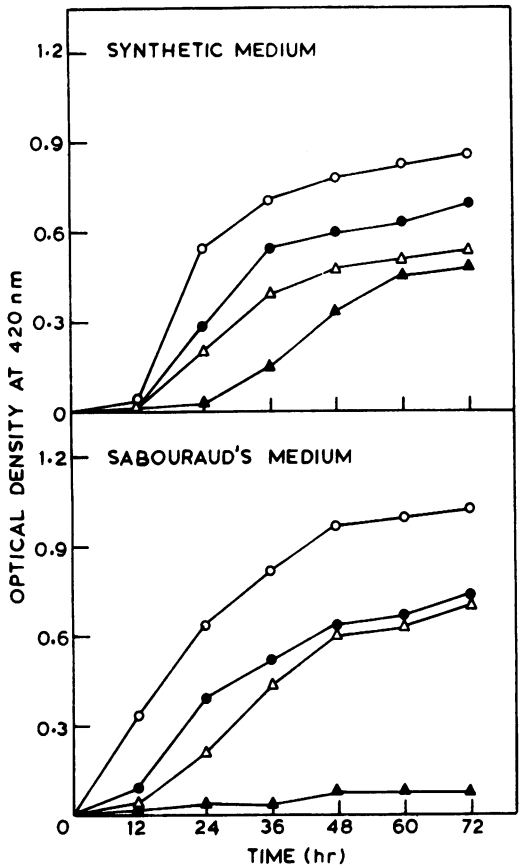

FIG. 2. *Effect of miconazole on the growth of Candida albicans. Symbols:* O, *control;* ●, *0.1 μg/ml;* Δ, *1.0 μg/ml;* ▲, *10.0 μg/ml.*

TABLE 2. *Effect of miconazole on viability of Candida albicans*[a]

Conc of miconazole (μg/ml)	Loss of viability at various incubation times (%)			
	2 h	4 h	8 h	12 h
0	0	0	0	0
2.5	31	60	81	83
5.0	34	61	85	91
10.0	69	97	99	100

[a] Cells of *C. albicans* (2×10^7 cells) (18 h old) were inoculated into 100 ml of Sabouraud glucose broth in 250-ml Erlenmeyer flasks and incubated on a rotary shaker at 30 C. Samples were removed at indicated time intervals and plated as described in Materials and Methods. Colony counts were made after 48 h of incubation at 30 C.

was high, the addition of glucose had not increased the oxygen uptake. Hence, the starved cells were used in glucose utilization. Glucose utilization was inhibited by 30% at 10 μg of drug per ml, and the inhibition exceeded over 80% at 100 μg of miconazole per ml. Total inhibition of glucose utilization was noticed at 500 μg of miconazole per ml.

Effect of miconazole on cell permeability. The leakage of various cellular constituents like 260-nm absorbing materials, proteins, and amino acids as affected by miconazole is shown in Fig. 4. There was very little leakage of 260-nm absorbing materials when cells were exposed to 25 μg of drug per ml. Concentrations above 25 μg/ml, however, brought about leakage, and it increased with time. But the maximum leakage occurred during the first 30 min of drug exposure. Appreciable amounts of leak-

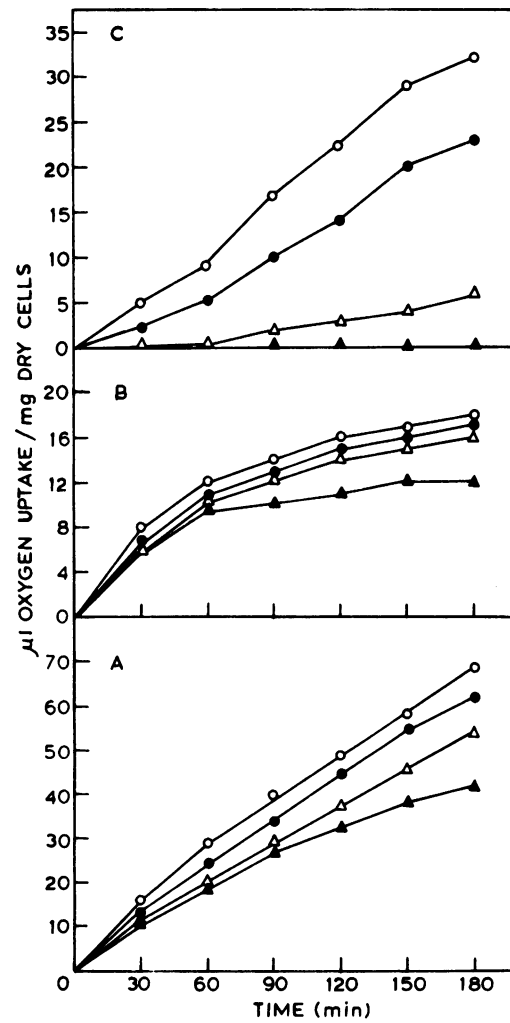

FIG. 3. *Effect of miconazole on respiration of C. albicans. Endogenous respiration of unstarved cells (A) and starved cells (B). Exogenous respiration (glucose)(C). Symbols:* O, *control;* ●, *10 μg/ml;* Δ, *100 μg/ml;* ▲, *500 μg/ml.*

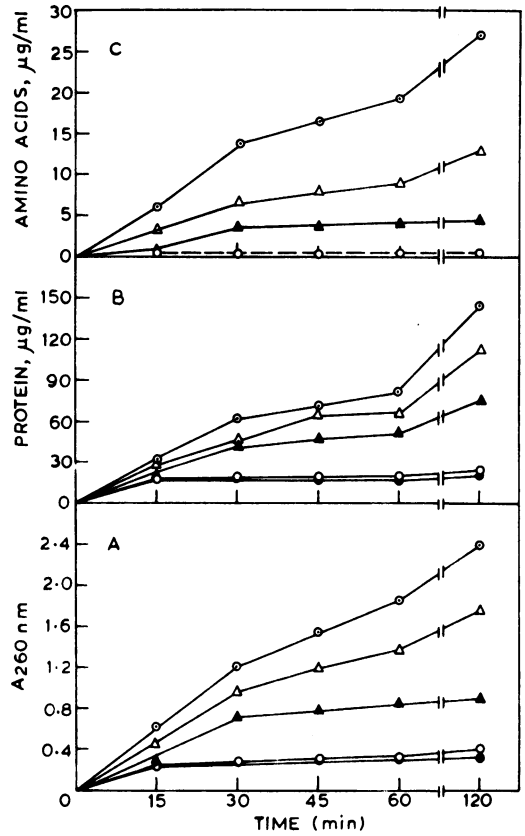

FIG. 4. *Effect of miconazole on the leakage of 260-nm absorbing materials (A), proteins (B), and amino acids (C) from the cells of C. albicans (1.5 × 10⁸ cells/ml). Symbols: ●, control; ○, 25 μg/ml; ▲, 50 μg/ml; △, 75 μg/ml; ⊙, 100 μg/ml.*

age continued to occur as the time of exposure to drug was prolonged. The leakage of proteins (B) also showed the same pattern as 260-nm absorbing materials. But at higher concentrations, the leakage increased with time, and the maximum leakage occurred within 30 min of drug exposure. Leakage of amino acids (C) also followed the same pattern as in the case of 260-nm absorbing materials and proteins.

The effect of drug on the leakage of potassium and sodium is shown in Fig. 5. The leakage of these two cations showed different patterns. Miconazole failed to cause any leakage of K^+ ions at 10 and 25 μg/ml. At 50 μg/ml the drug brought about marked efflux of K^+ ions. A small amount of Na^+ ions came out from cells of *C. albicans* in the absence of drug. In the concentration range of 10 to 25 μg/ml, the drug enhanced this leakage by two- to three-fold. Further increase in the drug concentration failed to bring about increased leakage.

The leakage of ^{32}P-labeled cellular constituents is shown in Table 3. The leakage is expressed in terms of counts per minute per milligram (dry weight) cells. The total ^{32}P taken up and incorporated by the cells during growth amounts to 3×10^5 counts per min per mg (dry weight) cells. From this data the percentage of leakage of ^{32}P-labeled cellular constituents was calculated. There was a very little leakage of labeled constituents in the absence of the drug. At 10 μg/ml the leakage was increased by 10-fold. Higher concentrations brought about

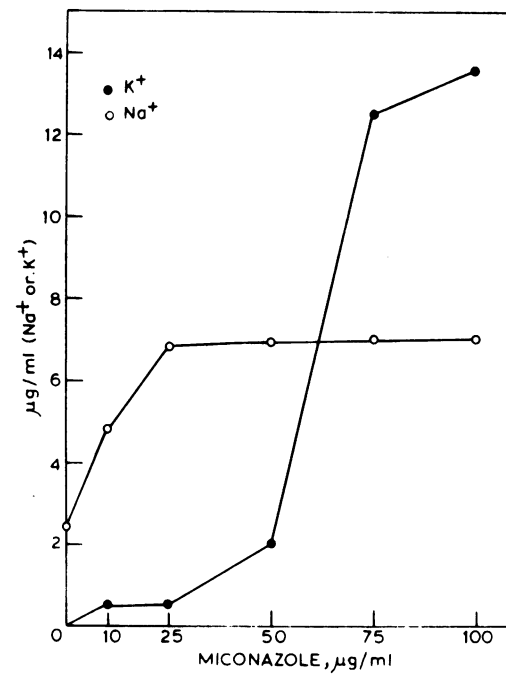

FIG. 5. *Leakage of Na^+ and K^+ from the cells of C. albicans (2.5×10^8 cells/ml) exposed to miconazole for 2 h.*

TABLE 3. *Effect of miconazole on the leakage of ^{32}P-labeled cellular constituents from the cells of C. albicans*

Miconazole (μg/ml)	Radioactivity (counts/min) in exudates from 1 mg (dry wt) of cells[a]	Leakage of ^{32}P-labeled compounds (%)
0	130	0.04
10	1,340	0.45
100	18,000	6.0
250	124,000	41.0
500	171,500	57.0
1,000	182,400	61.0

[a] Radioactivity of 3×10^5 counts per min per milligram (dry weight) of cells before exposure to drug.

an increasing amount of leakage. The leakage of 57% of the labeled constituents occurred at 500 μg/ml. A further increase of the drug by twofold, i.e., 1,000 μg/ml, has resulted in only slight increase in the effect.

Influence of divalent cations on miconazole effect. In synthetic medium where divalent cations like Mg^{2+} and Ca^{2+} are present, the growth inhibition of miconazole is less as compared in Sabouraud medium (Fig. 2). Whether these divalent ions have any effect on the miconazole action was examined by studying the drug effect on the leakage of 260-nm absorbing materials in the presence of increasing concentrations of either Ca^{2+} or Mg^{2+} (Fig. 6). Even at a very low concentration of Mg^{2+}, namely, 1 mM, the miconazole effect was reduced by approximately 34% and Ca^{2+} at the same level reduced the effect of miconazole by 46%. In the concentration range of 1 to 10 mM, Ca^{2+} exerted higher reversal than Mg^{2+}. At 10 mM level, both the metal ions caused a reversal of 82%, and further increase in the concentration of divalent ions had no increased effect.

DISCUSSION

Data presented in this study clearly reveal the potent growth inhibition exerted by miconazole on various species of *Candida*. Although all the species are highly susceptible to the drug, some, namely, *C. parapsilosis* and *C. pseudotropicalis*, are 1,000-fold more susceptible than *C. pelliculosa*, *C. guilliermondii*, *C. intermedia*, and *C. tropicalis*. The rapid uptake and stronger binding of the drug molecules by the highly susceptible strains are possibly responsible for these species differences.

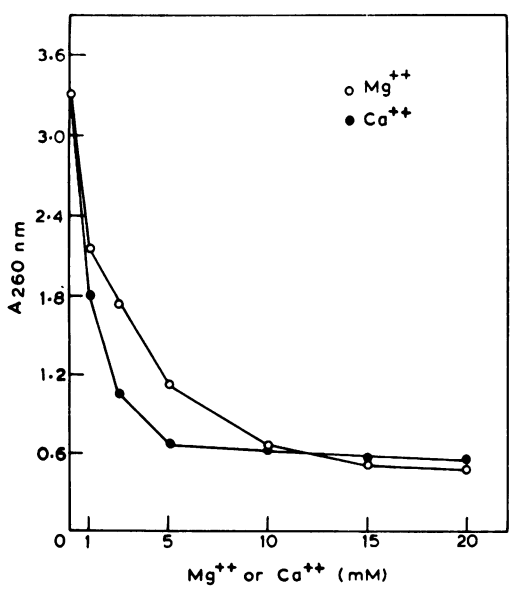

FIG. 6. *Reversal by Mg^{2+} and Ca^{2+} of the leakage of 260-nm absorbing materials from the cells of C. albicans (1.1 × 10^6 cells/ml) exposed to miconazole at a concentration of 100 μg/ml (0.2 mM).*

The activity of miconazole is affected by the media composition. In the synthetic medium, the decreased effect may be due to the reversal caused by the divalent metal ions like Ca^{2+} and Mg^{2+}. The leakage of 260-nm absorbing materials from cells of *C. albicans* is brought about by miconazole. This effect is greatly reduced in the presence of Ca^{2+} and Mg^{2+} ions. These divalent cations might compete with miconazole for binding sites, thereby reducing the effective concentration of drug to exert its effects.

Miconazole failed to affect the endogenous respiration, while greatly inhibiting utilization of glucose. A wide range of compounds such as amino acids, intermediates of citric acid cycle, and fats are known to be the sources of endogenous respiration in microorganisms. Hence, the lack of miconazole effect on endogenous respiration suggests its inability to interfere with the oxidative metabolism of these substrates. However, its potent inhibition of glucose utilization is probably at the level of substrate uptake by damaging the integrity of the cell membrane.

Literature is extensive in the area of membrane active antibacterial (1, 3, 7, 10, 14) and antifungal agents (8, 15). By affecting the membrane integrity and functions, they produce an initial rapid loss of high- and low-molecular-weight metabolites from the metabolic pool within the cell. Compounds like phenolics also initiate autolytic enzyme activity which cause extensive breakdown of proteins and nucleic acids. The hydrolytic products of these leak out from the cells in greater concentration. Antifungal antibiotics like polyenes specifically combine with sterol components in the membrane of susceptible organisms, resulting in the structural disruption of membrane and the loss of essential metabolites from the cell. The data presented here on miconazole show that the effect is on the cell membrane of the organism. There is very little autolytic action in the untreated cells. But miconazole brings about rapid loss of essential cellular constituents like proteins, amino acids, nucleotides and also monovalent cations. Its effect on membrane is also reflected in glucose utilization. The fact that miconazole is active on a wide range of gram-positive and gram-negative bacteria, besides yeasts and dermatophytes,

clearly reveals its binding to components present in all these structurally divergent microbes. The effect of miconazole is probably not by binding to a specific cell membrane component like ergosterol, the site where polyenes bind to exert their effect.

Antifungal agents exist which affect the cell membrane and its permeability, although their primary site of action has been proved to be elsewhere. Thus, lomofungin (6) inhibits the uptake of uracil and thymidine, and pyrrolnitrin (11) causes the leakage of 260-nm absorbing materials from the cells.

The present studies suggest the effect of miconazole on cell membrane; to exclude site(s) other than membrane, further studies are required.

ACKNOWLEDGMENTS

This work was sponsored by the Office of Naval Research, Washington, D.C., under contract no. N00014-71-C-0349. We express our grateful thanks to Ethnor Ltd., Bombay, for kindly supplying the miconazole sample.

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